mini-Tn*7* insertion in bacteria with multiple *glmS*-linked *att*Tn*7* sites: example *Burkholderia mallei* ATCC 23344

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The mini-Tn*7* vectors are universally applicable in Gram-negative bacteria and thereby facilitate the manipulation of many organisms for which few genetic systems are available. These vectors, when provided with only the Tn*7* site-specific transposition machinery, insert site and orientation specifically in the bacterial chromosome at an *att*Tn*7* site downstream of the essential *glmS* gene. A few bacteria, including *Burkholderia* spp., contain multiple *glmS* genes and therefore several *att*Tn*7* sites. Here we provide a protocol for application of the mini-Tn*7* system in *B. mallei* as an example of bacteria with multiple *glmS* sites. The procedure involves, first, cloning of the genes of interest into an appropriate mini-Tn*7* vector; second, co-transfer of the recombinant mini-Tn*7* vector and a helper plasmid encoding the Tn*7* site-specific transposition pathway into *B. mallei* by conjugation, followed by selection of insertion-containing strains; and last, PCR verification of mini-Tn*7* insertions. *B. mallei* possesses two *glmS* genes on chromosome 1 and Tn*7* transposes to both sites, although transposition to *att*Tn*7-1* associated with *glmS1* occurs in more than 90% of the clones examined. Transposition is efficient and the whole procedure from start to verification of insertion events can be done in less than 5 d. This first chromosome integration system in *B. mallei* provides an important contribution to the genetic tools emerging for *Burkholderia* spp. Vectors are available for gene complementation and expression, and gene fusion analyses.

INTRODUCTION

Burkholderia mallei is the etiological agent of glanders and a highly evolved obligate zoonotic mammalian pathogen that naturally affects horses, mules and donkeys[1,2]. *B. mallei* is highly infectious and results in a disease that is difficult to diagnose, hard to treat and often fatal[1,3]. Although glanders is a rare disease, *B. mallei* has received renewed attention because of its listing as a category B agent by the Centers for Disease Control as a consequence of increased concerns about biological weapons. A milestone in the research on this pathogen has been elucidation and publication of the *B. mallei* genome sequence, which has shown that the genome consists of two circular chromosomes[4].

Because no site-specific chromosome integration system currently exists for *B. mallei*, we sought to extend the use of the broad host-range mini-Tn*7* system[5] to this bacterium, which would make a valuable addition to the repertoire of genetic tools that are currently available (reviewed in ref. 1). A more detailed description of the mini-Tn*7* system and its features can be found in the accompanying protocol[6]. The mini-Tn*7* vectors are based on the well-characterized transposon Tn*7* (refs. 7,8; **Fig. 1a**). Unlike other transposons, Tn*7* integrates with high frequencies both site and orientation specifically at neutral Tn*7* attachment (*att*Tn*7*) sites. These sites are located downstream of the respective bacterial *glmS* genes, which encode essential glucosamine-6-phosphate synthetase[7,8]. Most bacterial genomes contain single *glmS* genes, and therefore most probably only a single *att*Tn*7* site. With the possible exception of *Burkholderia glumae*, which seems to contain a single *glmS* gene on chromosome 1 (I. Hwang, personal communication), *Burkholderia* spp. contain multiple *glmS* genes, and therefore multiple *att*Tn*7* sites (see **Fig. 1b**).

Burkholderia thailandensis contains two *glmS* genes, one on each of the two chromosomes[5]; *B. mallei* contains two *glmS* genes on chromosome 1; and the closely related *Burkholderia pseudomallei* contains three *glmS* genes, two on chromosome 1 and one on chromosome 2 (ref. 9). The presence of multiple *glmS* genes poses unique challenges for using the mini-Tn*7*

TABLE 1 | Currently available mini-Tn*7* vectors for *B. mallei* and intended uses[a].

Delivery plasmid and mini-Tn*7* element	Accession no.	Features and intended uses
pUC18T-mini-Tn*7*T	AY599230	Mobilizable base vector with MCS; for cloning of suitable selection markers or other functional and selectable elements
pUC18T-mini-Tn*7*T-Gm	AY599232	Gm^r on mini-Tn*7*T; mobilizable; MCS for gene cloning; for gene insertion in Gm^s bacteria
pUC18T-mini-Tn*7*T-Gm-REP	AY712952	Gm^r and *ori*$_{R6K}$ on mini-Tn*7*T; mobilizable; for determination of *att*Tn*7* sites in Gm^s bacteria

[a]Only those mini-Tn*7* vectors that are currently available for *B. mallei* for use with this protocol are listed. Although almost any of the Gm^r vectors described in **Table 1** of the accompanying paper[6] can be used with *B. mallei*, most of the delivery plasmids are pUC18 based and their use is therefore limited to transfer by electroporation. Similarly, the Gm^r marker does not work well with *B. pseudomallei* and *B. thailandensis*, and for these bacteria the zeocin and trimethoprim resistance markers, respectively, should be used instead. One should also keep in mind that in the United States usage of antibiotic resistance markers in select agents is restricted to those that do not interfere with clinical therapy. These rules may be different in other countries. Gm, gentamycin; MCS, multiple cloning site; r, resistant; s, susceptible.

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14. ABSTRACT

We recently constructed a series of broad-range mini-Tn7 vectors to address some of the problems inherent to plasmid-based cloning systems such as multi-copy state, necessity for continued selection and scarcity for many bacteria. The potential of the mini-Tn7 vectors for finding wide-spread biomedical and environmental applications, particularly for analysis of complex systems such as animal and biofilm models was demonstrated. In the presence of a helper plasmid encoding the site-specific transposition pathway, we showed that, with few exceptions, Tn7 insertions occured site- and orientation specifically at attTn7 sites downstream of bacterial glmS genes, with the number of possible insertions per genome roughly corresponding to the number of glmS genes present in these genomes. Our mini-Tn7 system is characterized by its versatility, simplicity, ease of use and ready adaptation to many bacteria. Here, we present protocols for use of our mini-Tn7 system in bacteria with single, multiple and secondary attTn7 sites.

15. SUBJECT TERMS

methods, broad range mini Tn7 vector, plasmid-based cloning

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system. Here we present a protocol for its use in *B. mallei* as an example of bacteria with multiple *att*Tn*7* sites. The mini-Tn*7* system greatly expands the genetic tools available for *Burkholderia* spp. and vectors have been developed that facilitate gene cloning and complementation, regulated expression from the *Escherichia coli tac* promoter, isolation of transcriptional or translational β-galactosidase (*lacZ*) fusions, and transcriptional bacterial luciferase (*lux*) fusions.

Table 1 lists mini-Tn*7* vectors currently available for use with *B. mallei* and includes tips for choosing the appropriate vectors for specific purposes. The ease of engineering diverse genetic traits into *B. mallei* will facilitate *in vivo* and *in vitro* studies with this bacterium, especially with animal model systems and biofilms, where antibiotic and plasmid selection are not feasible. The extension of the mini-Tn*7* system to other *Burkholderia* spp. and bacteria with multiple *glmS* genes is dictated by intrinsic susceptibilities and/or by select agent rulings (see **Table 1**, footnote a). Methods are provided for identification of *att*Tn*7* sites in bacteria for which only partial or no genome sequences are available.

MATERIALS

REAGENTS

- Bacterial strains (available from H.P.S.):
  - Helper strains SM10(λ*pir*)/pTNS2 and HB101/pRK2013
  - DH5α(λ*pir*) or CC118(λ*pir*)
  - *Burkholderia mallei* recipient strain; here strain ATCC 23344 (*B. mallei* distribution is limited to approved facilities and in the United States is governed by select agent rulings)
- DNA (plasmids available from H.P.S.):
  - Recombinant mini-Tn*7* suicide delivery vector DNA (for examples, see **Table 1**)
  - pUCGM (GenBank accession no. U04610)
  - pUC18 (ref. 10; commercially available from various vendors; e.g., Bayou Biolabs, cat. no. P-101)
- Oligonucleotides:
  - Gm-up (5′-TGGAGCAGCAACGATGTTAC-3′); 30 pmol μl^{-1}
  - Gm-down (5′-TGTTAGGTGGCGGTACTTGG-3′); 30 pmol μl^{-1}
  - P_{Tn7R} (5′-CACAGCATAACTGGACTGATTTC-3′); 30 pmol μl^{-1}
  - P_{Tn7L} (5′-ATTAGCTTACGACGCTACACCC-3′); 30 pmol μl^{-1}
  - $P_{BMGLMSD1}$ (5′-ACACGACGCAAAAGCGGAATC-3′); 30 pmol μl^{-1}
  - $P_{BMGLMSD2}$ (5′-AGTGGGCGTCGATCAACGCG-3′); 30 pmol μl^{-1}
- DMSO (e.g., Fisher Scientific, cat. no. D128-500)
- DNA isolation kits (e.g., QIAprep spin miniprep kit, Qiagen, cat. no. 27106, for plasmid DNA; and IsoQuick nucleic acid extraction kit, Orca Research, cat. no. MXT-020-100, for chromosomal DNA)
- DNA ladder (range 100 bp to 1 kb; e.g., the 50-bp DNA Step Ladder from Promega, cat. no. G4521)
- dNTPs (10 mM; e.g., Fermentas, cat. no. R0191)
- Gel extraction kit (e.g., QIAquick, Qiagen, cat. no. 28706)
- LB medium (e.g., EM Science, cat. no. 1.10285.5007)
- $MgSO_4$ (2 M and 10 mM; autoclaved; e.g., Fisher Scientific, cat. no. M63-500)
- NEBlot phototope kit (New England Biolabs, cat. no. N7550S)
- *Taq* polymerase and buffer (e.g., New England Biolabs, cat. no. M0267L)

EQUIPMENT

- Centrifuge tubes (15 ml, conical sterile, disposable with screw cap; e.g., Biologix, cat. no. 10-0152)
- Culture tubes (14 ml, 17 × 100 mm sterile, disposable with snap cap; e.g., VWR International, cat. no. 60819-761)
- Gradient cycler (e.g., Eppendorf Mastercycler gradient)
- Nitrocellulose filter membranes (25-mm diameter, 0.45-μm pore size; e.g., Schleicher & Schuell, cat. no. 10401106)
- Swinnex filter holders (25-mm; e.g., Millipore, cat. no. SX0002500)
- Syringes (3 ml, Luer lock; e.g., VWR, cat. no. BD512136)

REAGENT SETUP

Agarose gels Prepare agarose gels by adding agarose (e.g., ISC BioExpress, cat. no. E-3119) to TAE buffer[11] to achieve the desired agarose concentrations.

Antibiotic stock solutions Prepare stock solutions of ampicillin (100 mg ml^{-1}; e.g., Sigma Aldrich, cat. no. A9518), gentamycin (100 mg ml^{-1}; e.g., Sigma Aldrich, cat. no. G1264), kanamycin (50 mg ml^{-1}; e.g., Sigma Aldrich, cat. no. K4000) and polymyxin B (50 mg ml^{-1}; 100 mg ml^{-1}; e.g., Sigma Aldrich, cat. no. P0972) in sterile ddH_2O, filter-sterilize and store either frozen at −20 °C (ampicillin) or refrigerated at 4 °C (gentamycin, kanamycin and polymyxin B). Prepare 10 ml of the stock solutions and distribute 1.5-ml aliquots into sterile microcentrifuge tubes.

LB agar plates Using LB agar (e.g., EM Science, cat. no. 1.10283.5007), prepare LB agar plates, LB plates with 4% glycerol (LBG plates; add 40 ml of glycerol per liter of LB agar before autoclaving); LB plates with 100 μg ml^{-1} of ampicillin and 15 μg ml^{-1} of gentamycin (LB+Ap100+Gm15); LBG plates with 5 μg ml^{-1} of gentamycin and 15 μg ml^{-1} of polymyxin B (LBG+Gm5+Pxb15); and LBG with 10 mM $MgSO_4$ (spread 100 μl of sterile 2 M $MgSO_4$ onto LBG plate before conjugation). Expect 50 plates per liter of autoclaved agar.

PROCEDURE

Cloning of inserts

1| Clone the inserts into the multiple cloning site of the mini-Tn*7* vector of choice using standard cloning techniques[11]. The choice of vector will depend on what the intended applications of the final recombinant strains will be and some suggestions are given in **Table 1**.

Mini-Tn*7* delivery by four-parental mating conjugation ● TIMING 4d

2| Grow bacterial strains in sterile, disposable culture tubes at 37 °C overnight and with shaking at 225 r.p.m. in LB medium (or LBG for *B. mallei*) supplemented with the appropriate antibiotic (see REAGENT SETUP) as follows:

Strain	Selection
SM10(λ*pir*)/pTNS2	100 μg ml^{-1} ampicillin
HB101/pRK2013	35 μg ml^{-1} kanamycin
E. coli + mobilizable mini-Tn*7* delivery vector (e.g., pUC18T-mini-Tn*7*T-Gm; **Fig. 1**)	100 μg ml^{-1} ampicillin
Recipient strain	None

● TIMING ~15 min; end of day 1

! CAUTION *B. mallei* and other highly pathogenic bacteria must be handled with extreme caution and proper containment procedures must be used. Furthermore, in the United States *B. mallei* is classified as a select agent, and researchers and facilities handling select agents must be inspected, cleared and approved by the proper federal agencies before obtaining the agents and commencement of experiments. Pipette cultures carefully to avoid aerosolization of pathogenic bacteria and/or contamination of the pipette. Plugged pipette tips are recommended to avoid contamination of the pipette.

3| Owing to the risk of aerosol generation by electroporation, in our laboratories conjugation is the preferred means of DNA transfer with biosafety level 3 and other highly pathogenic agents. There are two options for setting up the conjugations on a filter membrane and they differ only in the methods by which the cells are combined and concentrated on the supporting filter membrane. Placing the conjugation mixture on a solid support facilitates *oriT*-mediated conjugation by immobilizing cells in close proximity to one another, and the solid support membrane also facilitates the recovery of cells. The filtration method is advantageous over the centrifugation method because it has fewer steps, thereby minimizing exposure to the pathogen.

Option A. Combining cells on a filter membrane using a syringe

(i) Pipette 100 µl of each saturated culture into a sterile, disposable culture tube containing 3 ml of 10 mM $MgSO_4$. Mix and filter through a 0.45-µm pore nitrocellulose filter, using a 25-mm Swinnex filter holder and a disposable, Luer lock, 3-ml syringe.

Option B. Combining cells on a filter membrane using centrifugation

(i) Pipette 0.1 ml of each culture into a microcentrifuge tube containing 0.6 ml of 10 mM $MgSO_4$. Centrifuge for 2 min at room temperature (20–25 °C) and reduced speed (7,000*g*). Reduced speeds yield softer cell pellets, which are easy to suspend and therefore minimize damage to cell-surface pili required for conjugation.

(ii) Carefully remove supernatant with a sterile, disposable, 1-ml pipette tip and dispose in biological waste; suspend cell pellet in 1 ml of 10 mM $MgSO_4$ and centrifuge as in Step 3Bi.

(iii) Remove and discard supernatant and suspend cell pellet in 30 µl of 10 mM $MgSO_4$.

4| Using alcohol- and flame-sterilized forceps, place the filter on a LBG plate supplemented with 10 mM $MgSO_4$ (see REAGENT SETUP) and incubate for 8 h at 37 °C.

▲ CRITICAL STEP Place the filter on an LBG plate and pre-warm at 37 °C for at least 30 min before adding cells to allow soaking of the filter with nutrients and to maximize conjugation efficiencies by avoiding a temperature 'shock'.

5| Using alcohol and flame-sterilized forceps, pick up and transfer the filter into a screw-capped, sterile conical tube containing 2 ml of PBS, and dislodge cells by vortexing for 30–60 s.

6| Spread 200-µl aliquots onto two LBG+Gm5+Pxb15 plates (see REAGENT SETUP). Incubate for 3 d at 37 °C.

● TIMING Steps 3–4, ~8.5 h; Steps 5–6, ~15 min; end of day 2.

? TROUBLESHOOTING

Check for insertions at *att*Tn*7* by colony PCR ● TIMING 3.5 h

7| Because the published *B. mallei* genome shows two *glmS* genes on chromosome 1 and therefore two *att*Tn*7* sites, two PCR reactions must be performed with each Gm^r transformant to be tested for insertions using a Tn*7*-specific primer coupled to different *glmS* downstream primers. Pick ten large colonies from Gm^r transformants obtained in Step 6. Transfer each colony to individual sterile microcentrifuge tubes containing 30 µl of sterile H_2O and boil for 5 min (use the recipient *B. mallei* strain grown on a LBG plate as a negative control).

▲ CRITICAL STEP Restreak the picked colonies on a LBG+Gm5+Pxb15 plate to maintain a stock for each screened colony.

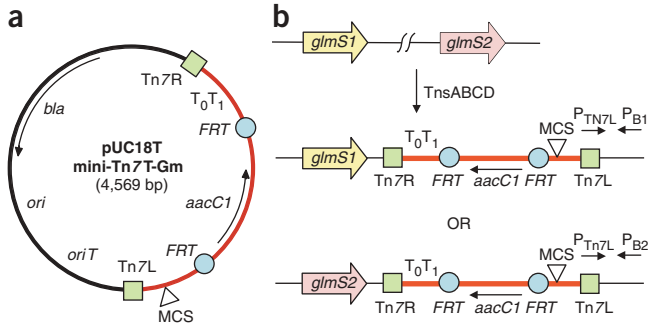

Figure 1 | Suicide delivery vector and integration of mini-Tn*7* at multiple *att*Tn*7* sites. (**a**) Map of a mobilizable pUC18-based suicide delivery plasmid containing mini-Tn*7*T-Gm. The extent of suicide vector and mini-Tn*7* DNA is indicated by the black and red line, respectively. Abbreviations: *aacC1*, gentamycin acetyl transferase–encoding gene; *bla*, β-lactamase–encoding gene; *FRT*, Flp recombinase target; MCS, multiple cloning site; *ori*, ColE1-derived origin of replication; *oriT*, origin of conjugative transfer; T_0T_1, transcriptional terminators T_0 and T_1 from bacteriophage λ and *E. coli rrnB* operon, respectively; Tn*7*L and Tn*7*R, left and right end of Tn*7*, respectively. Arrows indicate the extent and transcriptional orientation of the *aacC1* and *bla* genes. (**b**) Integration of mini-Tn*7* at two separate *att*Tn*7* sites on the same chromosome. In this example, mini-Tn*7*T-Gm is transposed into a bacterial chromosome (black line) containing two *glmS* genes after co-conjugation of pUC18T-mini-Tn*7*T-Gm and the pTNS helper plasmid encoding the TnsABCD transposase subunits, and Gm^r transformants resulting from transposition of mini-Tn*7*T-Gm (red line delimited by green squares) into the chromosome are selected. Owing to the high conservation of the *glmS1* and *glmS2* sequences, PCR for detection of insertion events is done with P_{Tn7L} and two different downstream bacterial primers, P_{B1} or P_{B2}, indicated by convergent arrows. Boxed arrows mark genes and their transcriptional orientations.

8| Pellet cell debris by centrifuging for 2 min at 16,000*g* and room temperature in a microcentrifuge. Transfer 25 µl of supernatant to a fresh microcentrifuge tube and place on ice.

9| To detect insertions at *glmS1* and *glmS2*, pipette the following ingredients into two separate sterile microcentrifuge tubes:

Component	Tube 1 Amount (µl)	Tube 2 Amount (µl)
ddH_2O	32.5	32.5
DMSO	2.5	2.5
10 mM dNTPs	1.0	1.0
10x *Taq* buffer	5.0	5.0
50 mM $MgCl_2$ (only if not included in *Taq* buffer)	1.5	1.5
Supernatant (from Step 8)	5.0	5.0
P_{Tn7L} (30 pmol μl^{-1})	1.0	1.0
$P_{BMGLMSD1}$ (30 pmol μl^{-1})	1.0	–
$P_{BMGLMSD2}$ (30 pmol μl^{-1})	–	1.0
Taq polymerase (5 U μl^{-1})	0.5	0.5
Total	50.0	50.0

10| Perform PCR as follows:

Cycle number	Denaturation	Annealing	Extension
1	95 °C, 5 min	None	None
2–31	95 °C, 45 s	59 °C, 30 s	72 °C, 20 s
32			72 °C, 10 min

■ **PAUSE POINT** PCR samples can be left in the PCR machine by including a holding step at 8 °C or they can be frozen indefinitely at −20 °C upon completion of the reactions.

11| Analyze 20-µl aliquots of the 50-µl PCR reactions by gel electrophoresis on a 2% agarose gel (see REAGENT SETUP) in TAE buffer (run at a constant 100–120 V for ~1 h). When using the P_{Tn7L}-$P_{BMGLMSD1}$ primer pair and the PCR cycle conditions indicated in Step 11, a single PCR fragment of 262 bp should be observed with DNA from Gm^r transformants from Step 6 (**Fig. 2a**, top). This fragment will be absent when using a DNA template derived from *B. mallei* ATCC 23344 (negative control). Although the primer pair P_{Tn7L}-$P_{BMGLMSD2}$ should yield a single fragment of 214 bp (**Fig. 2a**, bottom), it usually gives a fair amount of nonspecific amplification, but specificity is greatly enhanced in those strains containing Tn*7* insertions at *glmS2*. Further details are given in ANTICIPATED RESULTS. ● **TIMING** Steps 7–10, ~2.5 h; Step 11, 1 h; end of day 5.

? TROUBLESHOOTING

Methods for determination of integration sites of unknown sequence

12| In cases where multiple *att*Tn*7* sites are suspected in a bacterium for which either no or only partial genome sequence information is available, two options exist for determination of Tn*7* integration sites (**Fig. 3**). Option A involves a mini-Tn*7* containing a replicon that allows self-cloning of chromosomal DNA fragments containing this mini-Tn*7* insertion (**Fig. 3a**); Option B involves cloning of the chromosomally integrated antibiotic selection marker into an *E. coli* plasmid (**Fig. 3b**). We have used both options to determine unknown Tn*7* integration sites, but Option A is more straightforward, involving significantly fewer steps than Option B. Although the following procedures describe rescue of Gm^r markers, they can be applied equally to the rescue of any other antibiotic marker. These strategies can also be applied to the identification of secondary Tn*7* insertion sites that are not linked to *glmS* (for an example, see the accompanying *Proteus mirabilis* protocol[12]).

Option A. Use of a mini-Tn*7* containing a replicon ● **TIMING 7–9 d**

We have used this option successfully to determine the mini-Tn*7* insertion site in *B. thailandensis* E264 (refs. 5,13), and the protocol used for this bacterium is presented here, but similar procedures can be used for other bacteria. The procedure is shown in **Figure 3a**. Although gentamycin is not the best antibiotic for *B. thailandensis* because of the relatively high intrinsic resistance of this bacterium, it can be used at high concentrations (e.g., 450 µg ml^{-1}).

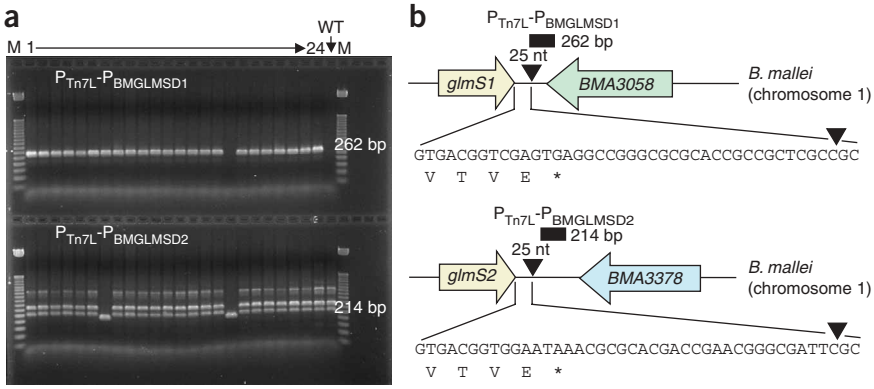

Figure 2 | Tn*7* transposition in a bacterium with two *glmS*-linked *att*Tn*7* sites: example *B. mallei* ATCC 23344. (**a**) PCR analysis of transposition events in *B. mallei*. Boiled preparative cell lysates were prepared from 24 Gm^r transformants obtained after conjugal transfer of the mini-Tn*7* suicide delivery vector pUC18T-mini-Tn*7*T-Gm (**Fig. 1a**) into *B. mallei* strain ATCC 23344. Chromosomal DNA contained in these lysates was used as a template in PCR reactions containing the indicated primer pairs, either P_{Tn7L}-$P_{BMGLMSD1}$ (top) or P_{Tn7L}-$P_{BMGLMSD2}$ (bottom), to detect insertions at *glmS1* or *glmS2*, respectively. A control DNA template derived from untransformed ATCC 23344 using the same boiling preparation was also included. Electrophoresis on a 2% agarose gel was used to analyze 20-µl aliquots of the 50-µl PCR reactions. Lanes labeled M contain molecular weight markers (here the 50-bp DNA Step Ladder from Promega); lanes 1–24 (left to right in both panels) contain PCR products obtained from 24 Gm^r transformants; lane 25 (labeled WT) contains the PCR products obtained from wild-type ATCC 23344. (**b**) Verification of mini-Tn*7* insertion sites by nucleotide sequencing. To determine the actual mini-Tn*7* insertion sites (triangles), transposon-chromosomal junction DNA sequences are determined by sequencing of the PCR fragments (horizontal bars labeled with the expected product sizes) amplified with the indicated primer pairs. Sequencing reactions are primed with either $P_{BMGLMSD1}$ or $P_{BMGLMSD2}$, taking into account the 5-bp duplication generated on Tn*7* transposition. *BMA3058* and *BMA3378* are *B. mallei* genes that encode proteins of unknown function. Boxed arrows mark genes and their transcriptional orientations. The sequences shown encompass the last five codons of the respective *glmS* genes and their immediate downstream sequences including the Tn*7* insertion sites (triangles).

(i) Use four-parental conjugation as described in Steps 2–6 to transpose a mini-Tn*7*T-Gm-REP (**Table 1**) containing the ori_{R6K} origin on the transposon into the chromosome of strain E264, but select transformants on LB medium with 450 µg ml^{-1} gentamycin.

(ii) Grow overnight cultures of a few Gm^r colonies in LB medium with 450 µg ml^{-1} of gentamycin at 37 °C. Using an appropriate method (e.g., an IsoQuick nucleic acid extraction kit), isolate genomic DNA from Gm^r colonies and digest 1 µg of DNA for 3 h at 37 °C with an enzyme (e.g., *Xho*I) that does not cut within the transposed sequences. Digestion with *Xho*I will yield a DNA fragment containing the whole transposon with the Gm^r marker and the ori_{R6K} origin, as well as flanking chromosomal DNA (**Fig. 3a**).

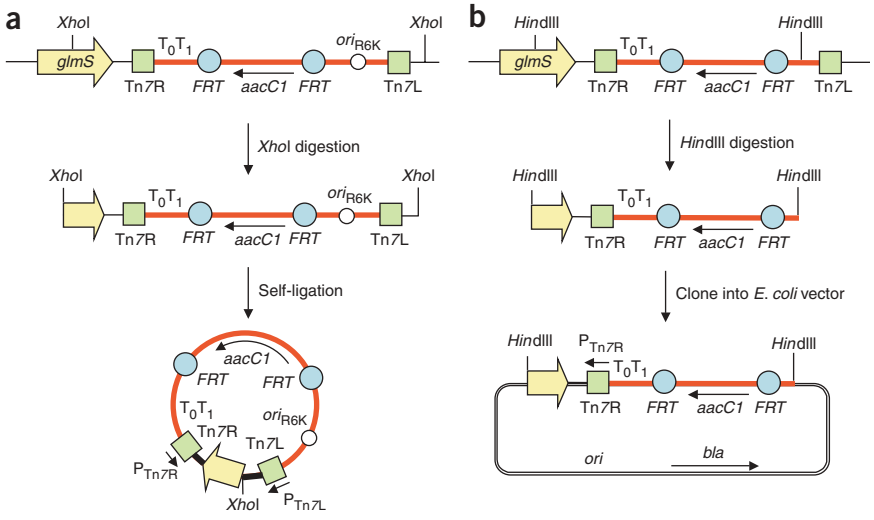

Figure 3 | Strategies for determination of Tn*7* insertion sites in bacteria with unknown sequences. (**a**) A mini-Tn*7* transposon containing a selectable marker (here Gm^r encoded by the *aacC1* gene) and a replicon (here ori_{R6K}) is integrated at the unknown *glmS*-linked *att*Tn*7* site. Chromosomal DNA is isolated and digested with an enzyme (here *Xho*I) that cleaves only in flanking chromosomal DNA (black lines), but not in the integrated mini-Tn*7* DNA (red line delimited by green squares). *Xho*I-digested DNA is then self-ligated and used to transform an *E. coli*(λ*pir*) strain capable of supporting replication of the plasmid resulting from self-ligation of the Gm^r and ori_{R6K}-containing fragment, and Gm^r transformants are selected. The nucleotide sequence of the *att*Tn*7* site is then determined by sequencing of the Tn*7*-chromosomal DNA junctions using P_{Tn7L} and P_{Tn7R}. (**b**) A mini-Tn*7* element containing a selectable marker (here Gm^r) is integrated at the unknown bacterial *att*Tn*7* site. Chromosomal DNA is isolated and digested with restriction enzymes that cleave the transposon once within its multiple cloning site (here represented by *Hin*dIII). Southern analyses using the Gm^r marker as a probe are then performed to identify a restriction enzyme yielding hybridizing DNA fragments of <6 kb. This enzyme (here *Hin*dIII) is used to cleave the chromosomal DNA, and fragments of the desired size range are purified from an agarose gel and ligated into an *E. coli* cloning vector and a selectable marker (here an ampicillin resistance-encoding gene, *bla*). The ligation mixtures are then transformed into an *E. coli* cloning strain and Ap^r+Gm^r transformants are selected. Finally, the nucleotide sequence of the *att*Tn*7* site is determined by sequencing of the Tn*7*-chromosomal DNA junctions using P_{Tn7R}. These two examples illustrate identification of *glmS*-linked *att*Tn*7* sites in bacteria with unknown genome sequences, but the same strategies can also be applied to identification of secondary Tn*7* insertion sites that are not linked to *glmS*. For abbreviations, see **Figure 1**.

(iii) Using standard methods[11], self-ligate chromosomal fragments and transform the ligation mixtures into *E. coli* CC118(λ*pir*) or DH5α(λ*pir*). Plate transformation mixtures on LB plates with 15 μg ml^{-1} of gentamycin to select ori_{R6K}-containing plasmids. Incubate at 37 °C until colonies appear.

(iv) Grow overnight cultures of 4–6 colonies in LB medium with 15 μg ml^{-1} of gentamycin at 37 °C. Isolate plasmid DNA using a commercially available kit (e.g., QIAprep spin miniprep kit).

(v) Verify presence of cloned sequences by restriction enzyme and agarose gel analysis, and determine chromosomal DNA-Tn*7* junction sequences by nucleotide sequencing using these plasmids as templates and P_{Tn7L} and P_{Tn7R} as sequencing primers.

(vi) After determination of chromosomal DNA-Tn*7* junction sequences, design primers to facilitate future detection of mini-Tn*7* insertions by PCR as described in Steps 7–11. ● **TIMING** Step 12Ai, 2–4 d (depending on growth rate of organism); Steps 12Aii–12Aiii, 2.5 d; Steps 12Aiv–12Av, 2 d.

Option B. Cloning of chromosomally integrated antibiotic resistance markers ● TIMING 9–11 d

We have used this procedure to clone the Gm^r marker, which was introduced into the chromosome via transposition of mini-Tn*7*T-Gm, but with appropriate modifications it can be used with other mini-Tn*7* elements. The procedure is shown in **Figure 3b**.

(i) Use four-parental conjugation as described in Steps 2–6 to transpose mini-Tn*7*T-Gm (**Table 1**) into the bacterial chromosome of interest.

(ii) Grow overnight cultures of a few Gm^r colonies in LB medium with gentamycin (concentration of antibiotic depends on the strain being studied) at 37 °C. Using the method described in Step 12Aii, isolate genomic DNA from Gm^r colonies and digest 1 μg of DNA for 3 h at 37 °C with various restriction enzymes that cleave the transposon once within the multiple cloning site (e.g., *Bam*HI, *Hin*dIII, *Kpn*I, *Xho*I, etc.). Digestion with these enzymes will generate a DNA fragment containing chromosomal DNA, the Gm^r marker and Tn*7*R (**Fig. 3b**).

(iii) Separate DNA fragments by agarose electrophoresis as described in Step 11, but use a 1% agarose gel, and then transfer DNA fragments to a nylon membrane by standard procedures[11].
▲ **CRITICAL STEP** Be sure to include a molecular size standard that can be detected with the method chosen for subsequent Southern blot analysis (e.g., biotinylated size markers should be used with biotinylated probes).

(iv) Prepare a Gm-specific probe by amplifying a 548-bp internal *aacC1* DNA fragment from pUCGM (GenBank accession no. U04610). To do this, set up PCR reactions as follows:

Component	Amount (μl)
ddH_2O	36.5
DMSO	2.5
10 mM dNTPs	1.0
10x *Taq* buffer	5.0
50 mM $MgCl_2$ (only if not included in *Taq* buffer)	1.5
pUCGM DNA (5 ng μl^{-1})	1.0
Gm-up (30 pmol μl^{-1})	1.0
Gm-down (30 pmol μl^{-1})	1.0
Taq polymerase (5 U μl^{-1})	0.5
Total	50.0

Perform PCR as follows:

Cycle number	Denaturation	Annealing	Extension
1	95 °C, 5 min	None	None
2–31	95 °C, 45 s	62 °C, 30 s	72 °C, 30–45 s
32	–	–	72 °C, 10 min

(v) Analyze PCR products by agarose gel electrophoresis as described in Step 11 and isolate the 548-bp Gm fragment from the gel by an appropriate procedure (we use a QIAquick gel extraction kit).

(vi) Label 75–100 ng of the purified Gm fragment by a method of choice. We biotinylate by random hexamer priming using the NEBlot Phototope kit. Use this labeled DNA fragment to probe the nylon membranes with the blotted chromosomal DNA fragments from Step 12Biii.

(vii) Analyze the blot from Step 12Bvi and digest 1 μg of DNA from Step 12Bii for 3 h at 37 °C with a restriction enzyme that yields a DNA fragment of <6 kb. This size is preferred because larger fragments are more difficult to clone.

(viii) Separate DNA fragments by agarose gel electrophoresis as described in Step 11, but use a 1% agarose gel. Excise the region of the gel containing the desired fragment size and extract DNA fragments as described in Step 12Bv.

(ix) Using standard procedures[11], ligate DNA fragments into an appropriate cloning vector (we have successfully used pUC18; ref. 10), and transform any competent *E. coli* cloning strain with the ligation mixture.

(x) Plate transformation mixtures on LB plates with 15 μg ml^{-1} of gentamycin and the antibiotic used to select for the cloning vector (e.g., 100 μg ml^{-1} of ampicillin for pUC18) to select recombinant plasmids containing the Gm^r marker.

(xi) Grow overnight cultures of 4–6 colonies in LB medium with 15 μg ml^{-1} of gentamycin at 37 °C. Isolate plasmid DNA using the method described in Step 12Aiv and verify presence of cloned sequences by restriction enzyme and agarose gel analysis. Determine chromosomal DNA-Tn*7* junction sequences by nucleotide sequencing using these plasmids as templates and P_{Tn7R} as the sequencing primer.

(xii) After determination of chromosomal DNA-Tn*7* junction sequences, design primers to facilitate future detection of mini-Tn*7* insertions by PCR as described in Steps 7–11.

● **TIMING**

Step 13Bi, 2–4 d (depending on growth rate of organism); Steps 12Bii–12Biii, 1 d; Steps 12Biv–12Bvi, 2 d; Steps 12Bvii–ix, 1 d; Steps 12Bx–12Bxi, 3 d.

? TROUBLESHOOTING

See **Table 1** for troubleshooting guidance.

TABLE 1 | Troubleshooting table.

PROBLEM	SOLUTION
Step 6 Conjugation does not work because of difficulties with counterselection and there is no concern about aerosols because the agent is not infective via this route.	Substitute published methods for preparation of competent cells and transformation. Co-transform target bacterium with mini-Tn*7* delivery vector and helper plasmid.
Step 11 The PCR does not work with a bacterium-specific primer or results in an unacceptable level of nonspecific DNA amplification even in the non-integrant control.	Most primers used in this protocol were designed with T_m values of 59–61 °C (calculated using the formula $T_m = [4(G+C)] + [2(A+T)] - 5$) and yield specific PCR fragments at annealing temperatures of 59–61 °C, but annealing temperature adjustments may yield improved results for some primers. However, complete elimination of nonspecific amplification usually requires synthesis of a new primer with similar placement, but different sequence. In some instances, there may be significant nonspecific PCR amplification in non-Tn*7*-containing strains, but specificity is greatly enhanced in those strains containing Tn*7* insertions.

ANTICIPATED RESULTS

Transposition efficiencies

Transfer of mini-Tn*7* into *B. mallei* using this procedure typically yields several hundred colonies per plate after 3 d at 37 °C. This number is more than sufficient for ready detection of insertions at both *glmS1* and *glmS2*. Numbers with other bacteria may be substantially lower but, owing to the high-frequency and high-fidelity insertion of mini-Tn*7* at defined *att*Tn*7* sites, large numbers of colonies are not required to achieve the desired results with almost any DNA transfer method.

Counterselection against the *E. coli* helper strains after conjugation is readily achieved by inclusion of polymyxin B into the selective medium, because *Burkholderia* spp. are naturally resistant to this antibiotic. With other bacteria and in cases where conjugation is the only choice for mini-Tn*7* delivery, alternative counterselection strategies may be exploited, such as intrinsic antimicrobial resistances or metabolic capabilities of the recipient that are absent from the *E. coli* donor strains. Alternatively, transformation can be used for mini-T*7* delivery[5] and this method obviously alleviates the need for counterselection.

Detection of mini-Tn*7* insertions

Once it has been established that mini-Tn*7* transposes to defined *att*Tn*7* sites in the bacterium of choice, PCR is a reliable method for verification of insertion events at any given *att*Tn*7* site. Bacteria that contain multiple copies of *glmS*, such as *Burkholderia* spp., also contain multiple *att*Tn*7* sites. Because of the high degrees of similarity of the *glmS* genes in these bacteria, a downstream primer in combination with P_{Tn7L} should be used to detect mini-Tn*7* insertions. Although it can be assumed that 100% of the antibiotic-resistant transformants contain mini-Tn*7* insertions, more than ten transformants should be analyzed to facilitate detection of insertions at all possible *att*Tn*7* sites.

Anticipated results for *B. mallei* ATCC 23344, exemplifying a bacterium with multiple Tn*7* insertion sites, are shown in **Figure 2a**. Most insertions are usually at one, preferred *att*Tn*7* site. In the case shown, 23 of 24 (96%) of the insertions are at the

*glmS1 att*Tn*7* site, as judged by the presence of a 262-bp PCR fragment (**Fig. 2a**, top, lanes 1–16 and 18–24) amplified with P_{Tn7L} and $P_{BMGLMSD1}$, which is absent in the recipient strain (**Fig. 2a**, lane 25). By contrast, only 8% (2 of 24) of the insertions are at the *glmS2 att*Tn*7* site, which is revealed by the presence of a 214-bp PCR fragment (**Fig. 2a**, bottom, lanes 7 and 17) amplified by P_{Tn7L} and $P_{BMGLMSD2}$. Only 1 of 24 (4%) of the transformants had insertions at both *glmS1* and *glmS2*, as judged by the presence of both the 214- and 262-bp PCR fragments (**Fig. 2a**, top and bottom, lane 7).

Similar results are also seen with *B. pseudomallei*, which contains three *glmS* genes: two on chromosome 1 and one on chromosome 2. Although mini-Tn*7* insertions are generally obtained at all three sites, insertions preferentially occur downstream of *glmS2* on chromosome 1 (Y. Casart, I. Beacham and H.P.S., unpublished results). The results obtained with $P_{BMGLMSD2}$ in **Figure 2a** also illustrate what is often seen during initial attempts at determining the *att*Tn*7* sites in any given bacterium. Even by increasing the annealing temperatures to 61 °C there may be a significant nonspecific amplification in non-Tn*7*-containing strains, but specificity is greatly enhanced in those strains containing Tn*7* insertions (**Fig. 2a**, bottom, lanes 7 and 17). Complete elimination of nonspecific amplification usually requires synthesis of a new primer with similar placement, but different sequence. We generally also verify the Tn*7* insertion sites by DNA sequencing of the PCR products from one or two insertions by priming the sequencing reactions with the different downstream primers, which for *B. mallei* were $P_{BMGLMSD1}$ or $P_{BMGLMSD2}$ (**Fig. 2b**).

Flp-mediated marker excision

Although all of our mini-Tn*7* vectors contain antibiotic selection markers that are flanked by Flp recombinase targets, Flp excision of chromosomally inserted markers in *B. mallei* and other *Burkholderia* spp. is currently hampered by the lack of a curable Flp source plasmid. Existing broad host-range Flp source plasmids rely on the *sacB* counterselection marker for curing[14], but *Burkholderia* spp., including *B. mallei*[4], contain endogenous *sacB* genes. Low-frequency excision of antibiotic-resistance markers could possibly be achieved by temporary Flp expression from a nonreplicative plasmid[15].

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